NO. 4. The Applicant's response concluded, therefore, that Adams, *et al.* is not prior art against this application.

The Examiner disagrees stating that the sequence Adams, *et al.* has a publication date of 1995 because this 294 nucleotide sequence of Adams, *et al.* was published in Nature, Volume 377 (6457 suppl.) pp 3-174. Consequently, Adams, *et al.* is prior art under 35 U.S.C.§ 102(b) and that when assigning effective filing dates to claims, each claim is considered as a whole.

Applicant will clarify. Base pair position 14-482 of SEQ ID NO. 4 in the instant application correlates with parent SEQ ID NO. 4 in its entirety. The claims originally filed in this application, as amended, claim 14-482 of instant SEQ ID NO. 4 and, therefore, entitled to the parent priority (i.e. October 31, 1996). New claim 49 covers instant SEQ ID NO. 4 in its entirety and is entitled the instant filing date.

SEQ ID NO. 5 is not found in parent case and is also included in new claim 49, which is entitled to the instant filing date. SEQ ID NO. 5 has been deleted from claims other than Claim 49.

Claims 1-10, 35 and 40-42 are rejected under 35 U.S.C. § 112, first paragraph because the claims have been amended to recite that the method is for detecting the presence of a polynucleotide indicative of breast disease by probing with a polynucleotide has 90% identity with SEQ ID NO. 5 and detecting the presence of the target polynucleotide indicative of breast disease. The Examiner concludes stating that the data presented in the specification does not associate breast disease with a marker that is 90% identical to this marker.

Applicant respectfully, yet vigorously disagrees. BS 106 is a mucin, which is a family of proteins which are valuable for their utility in diagnosing and monitoring cancer, and potentially as therapeutic targets in treating cancer. The fact that BS 106 is a member of the mucin familly is evidenced by many of its mucin-like properties, which are well known to those skilled in the art as being traits associated with mucins.

First, the BS106 protein sequence contains no N-linked glycosylation sites (NxS/T) but does contain multiple sites for potential O-linked glycosylation. According to the NetOGlyc 2.0 Prediction server (Center for Biological Sequence Analysis), which uses a neural network to predict mucin-type GalNAc O-glycosylation sites in mammalian proteins (Hansen et al, 1995, 1997, 1998), 15 of the 16 threonine residues in BS106 carry O-linked carbohydrate. Indeed, by ELISA assay, BS106m/h was found to bind to three

lectins including peanut lectin (Arachis hypogaea), osage orange lectin (Maclura pomifera), and jacalin lectin (Artocarpus integritolia) which all have recognition elements for galactose or N-acetyl galactose. BS106m/h did not bind to wheat germ lectin (Triticum vulgaris) which recognizes (GlcNAc)2, a disaccharide common to all N-linked sugar structures (data not shown). Thus, the above evidence illustrates BS106 to be a mucin due to the type of glycosylation and the large contribution this makes to the molecular weight.

Second, mucins are also characterized by the presence of a tandemly repeating sequence containing a high content of proline, threonine and/or serine residues within that sequence (PTS domains), as well as, extensive O-glycosylation of these threonine and/or serine residues. The tandemly repeating sequences are unique to each mucin and range in length from 8 amino acids (MUC5AC) to 169 amino acids (MUC6). BS106 contains such a tandemly repeating sequence, specifically, TTAAPTTA. This sequence is found three times, each time with a single amino acid substitution at the fifth position of the consensus sequence; alanine in the first repeat (res 46-53), proline in the second repeat (res 54-61), and serine in the third repeat (res 62-69). Again, this data illustrates that BS106 is indeed a mucin.

Third, mucins are characterized by a high molecular weight in the range of >100kD. BS106 itself has an observed molecular weight of approximately 40kD when analysed under denaturing and reducing conditions. BS106 contains one cysteine residue at the penultimate position of the mature sequence, providing opportunity for disulfide linkage with other molecules. Thus, BS106 'in vivo' is found as a high molecular weight species.

The importance of BS 106, a mucin, is its use as a diagnostic cancer marker. Mucins in general have been linked to cancer. For example, the enclosed review article entitled "MUC1 and Cancer" by Joyce Taylor-Papadimitriou et al (Exhibit A) reviews the subject of cancer and mucin detection. In particular, this article discusses a particular mucin, designated MUC1. BS 106 has similarities with MUC1. In particular, the tandem repeats and O-glycans pattern in BS 106 mentioned above are similar to those present in MUC1. MUC1, also known as CA15-3, has been used for cancer diagnostics. In fact, several diagnostics companies, have commercialized assays for CA15-3 in serum for the

detection of cancer. MUC1 is but one example of a mucin which is linked to cancer onset and progression.

In addition to BS106 being a mucin family member make it a good cancer diagnostic tool, but its utility to detect cancer is also illustrated by the data in the instant specification. As evidenced in Example 2, BS 106 is a novel polynucleotide which is found in the breast in women.. This marker and its corresponding EST's were found in 85.7% of breast libraries as compared to only 0.2% of non-breast tissue libraries. (Data obtained from Incyte Pharmaceutical's Lifeseq database). Therefore, this marker or fragments of this marker were found more than 389 times more often in breast than in non-breast tissue. As is known scientists skilled in the cancer diagnostic arts, a gene product, such as a protein or messenger RNA (mRNA) coding for the protein, which is more prevalent and specific to one tissue type than other tissue types, is extremely useful as a marker for the detection of disease in that tissue. If a protein appears in a tissue or body compartment where its normal occurrence is very low or non-existent, then the specific tissue in which the protein is normally found is in a diseased state. This is because the disease causes an alteration to the protein-specific tissue resulting in the protein escaping from its normal tissue into another. There are three main conditions which cause a tissue-specific protein to exist outside its specific host tissue: massive trauma, ischemia and hypertrophic proliferation. Thus, if a patient has not experienced a massive trauma or ischemia, detection of a tissue-specific protein outside that protein's host tissue indicates that the precise disease is hypertrophic proliferation of that tissue, the most serious form being cancer. There are many examples of the diagnostic use of tissue-specific protein markers . For instance, the appearance of prostate specific antigen (PSA) in the prostate and seminal plasma is normal, but its detection in blood is indicative of prostate cancer. Further, the appearance of PSA messenger RNA (mRNA) in blood is indicative of prostate cancer. Likewise, the appearance of carcinoembryonic antigen (CEA) in colon and stool is normal, but its detection in blood at elevated levels is indicative of colorectal cancer. Thus, the appearance of BS 106 protein or mRNA in a patient blood sample is indicative of breast disease .

The claims have been amended such that the polynucleotide encodes for a mucin and is 90% identical to the Sequence ID No listed thereby characterizing the claimed polynucleotides. Thus, based on the above remarks and amendments, it is requested that the rejection based on 35 U.S.C. 112 be withdrawn.
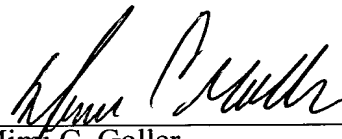
CONCLUSION

In view of the aforementioned amendments and remarks, the aforementioned application is in condition for allowance and Applicant requests that the Examiner withdraw all outstanding objections and rejections and to pass this application to allowance.

Respectfully submitted,

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Review

MUC1 and cancer

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Abstract

The MUC1 membrane mucin was first identified as the molecule recognised by mouse monoclonal antibodies directed to epithelial cells, and the cancers which develop from them. Cloning the gene showed that the extracellular domain is made up of highly conserved repeats of 20 amino acids, the actual number varying between 25 and 100 depending on the allele. Each tandem repeat contains five potential glycosylation sites, and between doublets of threonines and serines lies an immunodominant region which contains the epitopes recognised by most of the mouse monoclonal antibodies. The O-glycans added to the mucin produced by the normal breast are core 2 based and can be complex, while the O-glycans added to the breast cancer mucin are mainly core 1 based. This means that some core protein epitopes in the tandem repeat which are masked in the normal mucin are exposed in the cancer associated mucin. Since novel carbohydrate epitopes are also carried on the breast cancer mucin, the molecule is antigenically distinct from the normal breast mucin. (Changes in glycosylation in other epithelial cancers have been observed but are not so well documented.) Immune responses to MUC1 have been seen in breast and ovarian cancer patients and clinical studies have been initiated to evaluate the use of antibodies to MUC1 and of immunogens based on MUC1 for immunotherapy of these patients. The role of the carbohydrates in the immune response and in other interactions with the effector cells of the immune system is of particular interest and is discussed. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Membrane mucin; Breast cancer; O-glycan immunotherapy

Contents

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1. Historical background

The epithelial mucin which is coded for by the MUC1 gene is not a classic extracellular complex mucin such as those found as major components of the mucous layers covering the gastro-intestinal and respiratory tracts, but is a transmembrane molecule, expressed by most glandular epithelial cells. The molecule was first identified in human milk (where it is shed from the milk secreting epithelial cells), as a large molecular weight glycoprotein rich in serines, threonines and prolines carrying a high percentage of O-linked carbohydrate [1]. It is probably fair to say, however, that interest in the molecule was dramatically enhanced by cancer researchers using the then newly developed monoclonal antibody technique to identify surface molecules on normal epithelial cells, and the carcinomas which develop from them. Since the molecule is highly immunogenic in the mouse, many antibodies were developed to the MUC1 mucin by various groups using normal or malignant epithelial cells, or their membranes, as immunogens. In particular, the membrane of the lactating mammary epithelial cell which surrounds the fat globules shed into milk was widely used as an immunogen, where the MUC1 mucin apparently dominates in inducing antibody responses. The antibodies to this molecule have been analysed in two 'wet' workshops [2,3] as well as by individual investigators [4–6] so that the epitopes which they recognise have been extremely well characterised. Many map to a region between pairs of serines and threonines within the tandem repeat (see Fig. 1).

From immunohistochemical staining of tissue sections with the antibodies it became clear that although MUC1 is widely expressed by normal glandular epithelial cells [7], the expression is dramatically increased when the cells became malignant. This has been well documented in breast and ovarian cancer [8] and available data suggest this is also true in some lung, pancreatic and prostate cancers [8–10]. The antibody studies also gave the first indications that not only is the mucin over-expressed in carcinomas, but the pattern of glycosylation is altered. Thus in the breast cancer mucin, glycosylation changes result in certain epitopes in the core protein being exposed which are masked in the mucin produced by the lactating mammary gland [11]. The antibody SM3 [12] has been particularly important in defining this difference, the epitope in the tandem repeat being accessible in breast cancers, but not in normal breast [8]. Changes in glycosylation patterns also occur in other malignancies, as in the colon [13]. However, the changes are imposed on the normal pattern of glycosylation, which can be very different in different tissues.
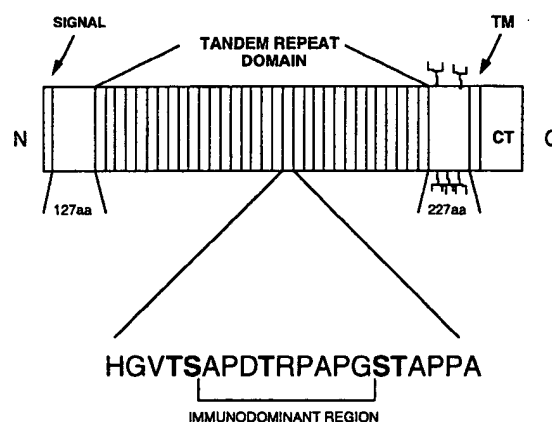

Fig. 1. Core protein of the MUC1 mucin. The amino acids in bold refer to potential O-linked glycosylation sites. ─[= potential N-linked glycans.

Because of the availability of antibodies to the core protein, the gene coding for this protein was the first mucin gene to be cloned [14,15]. The extracellular domain was found to be made up of tandem repeats (TRs) of 20 amino acids each of which contain five potential O-glycosylation sites, and TR domains have been found to be a common feature of all the mucin core proteins for which sequence has been obtained. A similar strategy of raising antibodies to the core protein stripped of carbohydrate was used to clone the cDNAs encoding the core protein of other mucins. As predicted from previous work, the gel-forming extracellular mucins (such as MUC2 and MUC3) are more complex, containing cysteine rich domains which are involved in oligomerisation as well as the TR domains for attachment of O-glycans [16]. MUC4 has recently been fully sequenced and identified as the second epithelial mucin which is a transmembrane molecule [17], and homologous to the rat sialomucin characterised by Carraway and colleagues [18]. In the context of target antigens in cancer, membrane mucins have the advantage of being accessible to cytotoxic antibodies: The antibodies to the MUC1 mucin have been available since the early 1980s and clinical studies with these antibodies are beginning to mature. With the availability of the cDNA coding for the core protein of the MUC1 mucin, and the characterisation of the changes in glycosylation which can occur in cancer, focus is now also being given to the use of antigens based on MUC1 in 'active specific immunotherapy, of cancer patients.

The studies on MUC1 have developed in such a way as to bring together investigators from several disciplines including glycobiology, immunology and oncology. In this article, we will attempt to integrate data from these different areas by: (1) reviewing the data documenting the changes in glycosylation of MUC1 which can occur in cancer (which also have relevance to basic changes in O-glycosylation patterns in malignancy); (2) discussing the relevance of the over-expression and altered glycosylation of MUC1 to malignant disease and the immune response; and (3) summarising the clinical studies which are being done using antibodies and antigens based on MUC1 to immunotherapy of cancer patients.

2. Changes in glycosylation of the MUC1 mucin in cancer

2.1. The MUC1 core protein

Fig. 1 shows a diagram of the core protein of the MUC1 mucin indicating the sequence of the tandem repeat as deduced from the nucleotide sequence. The extracellular domain is made up largely of 20 amino acid tandem repeats, the number of which varies with the allele, making the gene highly polymorphic [19]. The TR domain contains multiple potential O-glycosylation sites and is flanked by degenerate tandem repeats which also contain such sites, while there are five potential N-glycosylation sites closer to the transmembrane domain [14,15]. Comparison with the homologous gene in other species shows a high conservation of sequence in the transmembrane and cytoplasmic domains, while within the tandem repeats conservation is limited to retaining the sequence as a scaffold for the addition of O-glycans [20]. The function of the highly conserved regions has not been defined, but it is suggested that these domains may play a role in signal transduction and in protein interactions. A shorter molecule coded for by a mRNA where the TR domain has been spliced out may be more involved in these functions and has been reported to be preferentially expressed in tumours [21]. In the context of analysing the glycosylation patterns of MUC1 and its role in inducing an immune response, clearly the full-length molecule, which is the dominant form is of central importance.

2.2. O-Glycosylation in mammalian cells

The addition of N-acetyl galactosamine to serine or threonine initiates O-glycosylation in mammalian cells [22]. It is now clear that this reaction is catalysed not by one, but by a family of enzymes (polypeptide N-acetylgalactosaminyltransferases) which show overlapping but different specificities with regard to peptide sequence [23]. The three GalNAcTs (GalNAcT1, T2, and T3) which have been specifically localised by immunoelectromicroscopy in cells transfected with the tagged genes, are found distributed throughout the Golgi apparatus [24], indicating

that chain initiation may not be restricted to the *cis* compartment. *O*-Glycans are then synthesised by the addition of sugars individually and sequentially as the mucin passes through the Golgi. Chains are generally extended by the addition of polylactosamine side chains and terminated by the addition of sialic acid, fucose or galactose. Again it is becoming clear that more than one enzyme can catalyse the same specific reaction, and that the same substrate may be acted on by enzymes which produce different products. Thus the *sites* of glycosylation of the same core protein could vary depending on the profile of expression of GalNAcTs, while the *composition* of the *O*-glycan will be influenced both by the level of activity of a specific glycosyltransferase, and its position in the Golgi pathway relative to other enzymes which can compete for the same substrate. The early work with antibodies indicated that there were differences in the pattern of *O*-glycosylation of MUC1 in breast cancer. These changes could involve differences in sites of glycosylation or in *O*-glycan structure or both.

2.3. Changes in the sites of O-glycosylation of MUC1 in breast cancer

The techniques for analysing specifically the sites in proteins which are *O*-glycosylated in vivo have only recently been developed to the point where they can be applied to mucin molecules which may contain hundreds or even thousands of *O*-glycans [25]. There are five potential *O*-glycosylation sites in each tandem repeat of MUC1 and the use of these sites in the normal and malignant mammary gland has recently been analysed. Analysis of the mucin produced by the normal lactating mammary gland has shown that while all of these sites can be glycosylated, the *average* number of *O*-glycans added to each tandem repeat is around 2.5 [26]. Analysis of the sites of glycosylation of the MUC1 mucin produced by the breast cancer cell line T47D, however, suggests that all the five sites are occupied in all the tandem repeats (F.G. Hanisch, personal communication). This difference could be attributable to a different profile of expression of the GalNAcTs in normal and malignant mammary tissue. However, initial analysis of expression of four GalNAcTs in sections of breast cancers by immunohistochemistry has not
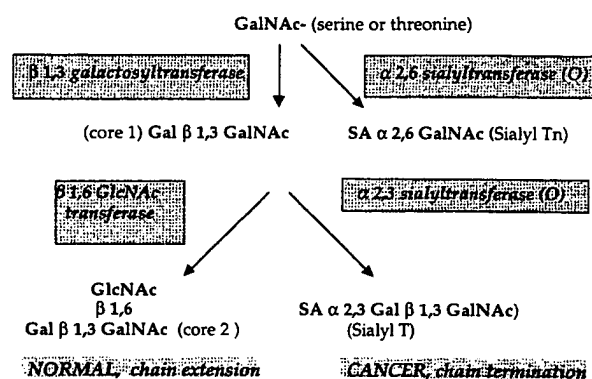
Fig. 2. Pathways of *O*-glycosylation of MUC1 in breast tissue. In normal mammary epithelial cells core 1 glycans are converted to core 2 by the action of core 2 β1,6GlcNAcT. In breast carcinomas, this conversion is reduced, resulting in core 1-based structures being found on MUC1.

given a clear answer to this point (Burchell and Mandel, unpublished observations). Another possible explanation is that the *O*-glycans on the T47D mucin which are shorter (see below) allow initiation of glycosylation to proceed throughout the Golgi apparatus, while the longer side chains which are built on to the normal mucin block access of the GalNAcTs at a certain point.

2.4. Changes in the composition of O-glycans added to MUC1 in breast cancer

In the normal human mammary gland, the addition of GalNAc to serines or threonines is followed by the addition of galactose to form core 1 which then acts as a substrate for the core 2β1,6GlcNAc T (C2GnT) enzyme, leading to the formation of core 2 (see Fig. 2). Type II polylactosamine side chains are then formed and terminated by sialic acid or fucose [27,28]. Thus the *O*-glycans on the normally glycosylated mucin are core 2 based. To analyse the structure of the *O*-glycans attached to MUC1 produced by breast cancer cells, the mucin has been labelled, either internally with ^{3}H-glucosamine hydrochloride [29], or exogenously with tritiated borohydride [30], and purified with antibodies to the mucin. Released labelled *O*-glycans were then analysed by comparison to standards in Dionex chromatography. The internally labelled mucin was precipitated from T47D cells using a polyclonal antibody to the cytoplasmic tail which recognises all glycoforms [31]. The exoge-

nously labelled MUC1 was purified from the breast cancer cell line, BT20, using immuno-affinity chromatography and a monoclonal antibody (DF3) directed to an epitope in the tandem repeat which could select for certain glycoforms. In both cases, however, the structure of the *O*-glycans was found to be core 1 and not core 2 based. In the case of T47D, 59% of the glycans had the structure of the disaccharide Galβ1,3 GalNAc, i.e the T blood group antigen, 33% consisted of sialyl T, while a small proportion, 8%, was unsubstituted GalNAc (Tn). These direct analyses confirm the earlier work of the late George Springer, who demonstrated the appearance of the T epitope in breast cancer [32] and used a preparation of erythrocytes expressing this antigen in the treatment of breast cancer patients. The more recent data related to MUC1 identify a core protein which carries this *O*-glycan. The sialyl Tn epitope (NeuAcα2,6 GalNAc) has been reported to be specifically expressed in several cancers, including gastric and breast, and has been used, coupled to a carrier protein as an immunogen for cancer patients (see below). However, sialyl Tn was not found in any quantity in the studies analysing the *O*-glycans on MUC1 mucin from breast cancer cell lines.

2.5. Correlation in changes of glycosyltransferase activities with changes in O-glycan structure in breast cancer

An examination of Fig. 2 shows that core 1 can be a substrate for an enzyme leading to chain extension via core 2 or for an enzyme which, by adding sialic acid to core 1 to form sialyl T, terminates chain extension. Analysis of the activity of the enzymes catalysing these reactions in extracts of normal and malignant breast epithelial cell lines showed that the α-2,3-sialyl-transferase activity was increased 8–10-fold in three breast cancer cells lines relative to the normal cell line (MTSV1–7), while the C2GnT enzyme activity was absent in two of the breast cancer cell lines (T47D and BT20) and reduced by 50% in a third [33]. Observations at the mRNA level revealed that C2GnT mRNA was essentially absent in the T47D and BT20 lines. However, C2GnT mRNA was paradoxically elevated in the MCF-7 line suggesting translational/post-translational control. Analysis of C2GnT promoter activity via 5′-untranslated

regions reveal no significant alternative promoter usage in any of the cell lines (Dalziel et al., unpublished observations) although evidence for multiple promoters was detected with the mouse C2GnT [34].

Clearly in measuring the activity of a cell extract several enzymes may be involved in the same reaction. In the case of the activity, catalysing the formation of core 2 from core 1, it is likely that the L enzyme which has been cloned [35] is being measured as the activity of the M enzyme which also catalyses the formation of core 4 [36], was not detected in these cells [33]. In the case of the sialyltransferase, there are at least three enzymes which could theoretically catalyse the formation of the addition of sialic acid in α2,3 linkage to Galβ1,3-GalNAc [37,38]. However, in situ analysis of tumour sections with a specific ST3Gal I probe [39] shows a marked increase in expression of this specific sialyltransferase in breast cancers. Significantly the increased expression correlated with both the invasiveness of the tumour, and with the expression of the SM3 epitope.

The levels of mRNA coding for the c2GnT were not consistently decreased in the breast cancers examined by in situ hybridisation with the C2GnT probe. However, if the two enzymes are in a similar position in the Golgi then increasing the activity of one could compete effectively for the common substrate, i.e. core 1. The availability of the cloned genes makes it possible to transfect cells and thus (a) alter the level of enzyme expressed and (b) by using a gene tagged with an epitope recognised by an antibody, localise the enzyme in the Golgi apparatus. Such experiments have shown that the ST3Gal I sialyltransferase is mainly located in the medial/*trans* Golgi with some being detected in the *cis* Golgi compartment, and that over expressing the enzyme in the normal MTSV1–7 cells results in a reduction in the core 2 based structures added to MUC1 [40]. Localisation studies with the C2GnT suggest that it is mainly in the *cis*/medial Golgi; MUC1 from C2GnT transfected T47D cells has core 2 structures (instead of the core 1 structures present in the untransfected cells) (Whitehouse, Dalziel, Burchell and Taylor-Papadimitriou, manuscript in preparation). Significantly, the T47D cells over expressing the core 2 enzyme lose expression of the SM3 epitope which is exposed in the untransfected cells. Thus,

although the changes in the composition of O-glycans added to MUC 1 in breast cancer may not be completely explained by the increase in sialyltransferase activity, (since a high proportion of the O-glycans on the cancer mucin are not sialylated), the enzyme does appear to compete with the core 2 enzyme for core 1 and high levels of activity are associated with the expression of the tumour-specific SM3 epitope.

It should be noted, of course, that the changes in expression of glycosyltransferases seen in breast cancer will affect the glycosylation not only of MUC1, but also of other molecule undergoing mucin-type O-glycosylation.

2.6. Changes in glycosylation of MUC1 in other cancers

Human colon cancer is associated with antigenic and structural changes in mucin type O-glycan carbohydrate chains. In particular loss of O-glycans has been reported [41]. Changes in the carbohydrate epitopes carried on MUC1 have been detected in colon cancer by purifying components from cancer cell lines which express the epitopes sialyl Le^x and Le^a which are not well expressed in the normal colon [42]. The full structures have been analysed, but the direct comparison with the full structures on the cancer associated mucin carried on the mucin produced by normal colon has not been done. However, it is clear that the SM3 epitope, which is absent from normal colon can be exposed in some colon cancers [8]. This epitope also appears in ovarian cancers [8] and gastric carcinomas [43], suggesting some modification of the side chains in these diseases.

The increased expression of the sialyl Le^a epitope in colon cancer is of interest as this epitope is expressed on the glycoform of the selectin ligand PSGL1 which interacts with P and E selectin. Data from Hansson and colleagues [42] show that MUC1 carrying sialyl Le^a and Le^x which was purified from a colon carcinoma cell line, inhibited the adhesion of a leucocyte cell line to cells transfected with E-selectin (see below). Interestingly, the increase of sialyl Lewis^x expression in colon carcinomas has been attributed to a decrease in O-acetylation of mucin-bound sialyl Le^x which occurs in colon carcinomas [44].

3. Expression of MUC1 and malignant progression

3.1. Effects of MUC1 expression on the behavioural properties of cancer cells

3.1.1. Effects on cell interactions and tumourogenicity

The changes in expression and post-translational modification of MUC1 has stimulated investigations into whether this affects the behaviour of cancer cells, particularly in relation to interactions with other cells and with the extracellular matrix. In the normal glandular epithelial cell, MUC1 expression is limited to the apical surface bordering a lumen. In cancer cells however, which have lost polarity, the mucin is expressed all over the surface. Because of its rod-like structure, the molecule extends more than 100–200 nm above the surface, which is 5–10-fold the length of most membrane molecules. By virtue of the high level of sialic acid, MUC1 is also negatively charged and cells expressing high levels may repel each other. Such repulsive effects have been demonstrated by showing that MUC1 transfectants show reduced aggregation as compared to the non-expressing parental cells [45] and interactions with the extracellular matrix are also inhibited [46]. With E-cadherin-mediated cell interactions, MUC1 has been reported to be inhibitory (in L cells transfected with E-cadherin and MUC1) [47], and to enhance adhesion by interacting with β-catenin [48].

In considering the effects of MUC1 on cell–cell interactions, it is clear that without specific interactions, for example with a lectin molecule, the long highly charged molecule can easily result in repulsion between cells. These inhibitory effects on cell interactions appear to depend on both the large size of the molecule and the negative charge [46,47]. However, where a specific interaction is possible, for example a particular carbohydrate epitope binding to a lectin, then cell interactions may be enhanced. Thus, MUC1 has been reported to be a ligand for ICAM1 expressed by endothelial cells [49] and to enhance antigen presentation to T-cells, possibly operating through a lectin interaction [50]. Furthermore, MUC1 has been shown to be a ligand for sialoadhesin, a macrophage restricted adhesion molecule, which specifically binds Neu5Aα2,3Gal and so may be involved in recruiting macrophages into the tumour site [51].
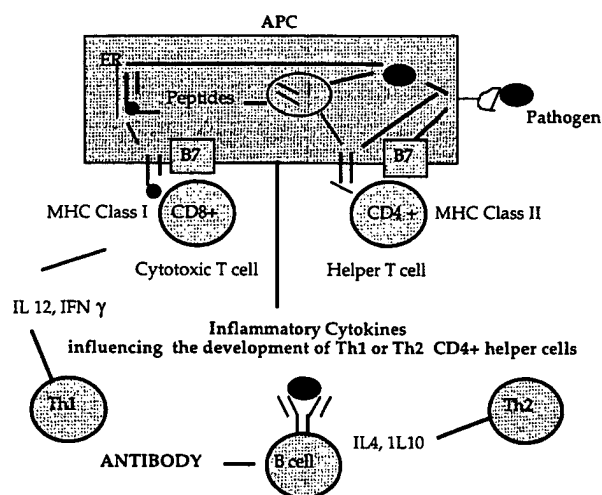
Fig. 3. Recognition of a pathogen by APCs through innate immunity leads to activation of adaptive immunity.

When the glycosylation pattern of the mucin is changed in carcinomas, resulting in the production of different glycoforms, such carbohydrate dependent interactions will be affected. The O-glycans on the cancer mucin will also vary with the specific carcinoma, so that while selectin ligands may appear on MUC1 produced by colon carcinomas, these may not be present in breast cancers. How the surface MUC1 on cancer cells may influence metastatic progression is not clear, although in Muc-1 null mice, mammary tumour progression has been reported to be delayed [52].

3.1.2. MUC1 expression and immune responses

Cell–cell interactions in the context of cellular immune responses are of particular interest, since MUC1 is under intensive study as a possible immunogen for immunotherapy of some cancer patients. Before considering these studies, it is appropriate to consider briefly the framework of the different compartments of the immune response to see how these might be effectively recruited by MUC1 based immunogens to kill cancer cells.

The immune system was developed to reject invading pathogens, be these extracellular as with most bacteria and fungi, or intracellular parasites such as viruses. In vertebrates, there are two major compartments namely the 'innate' immune response and the 'adaptive' immune response. Lower organisms use

only innate immunity where molecules with a specific structural pattern, characteristic of a class of pathogens (pathogen associated molecular patterns or PAMPs) are recognised [53]. Examples of these are the mannans of yeasts, lipopolysacharides of Gram-negative bacteria or bacterial DNA which contains unmethylated CpG dinucleotides [54]. Many of these PAMPs are rich in carbohydrates and can interact with lectins. Their interaction with lectins on antigen presenting cells (APCs) activates the cells to increase expression of molecules (MHC, co-stimulatory molecules and cytokines) required to stimulate the adaptive immune response mediated by T-cells and B-cells (see Fig. 3). These cells recognise small domains or 'epitopes' which, in the case of B-cells are present in a large molecule or antigen and recognised by surface immunoglobulin. T-cells, however, recognise peptides derived from the larger molecule which are presented either by MHC class I molecules, which activate the T-cell receptor (TCR) of CD8+ cytotoxic T-cells (CTL), or by MHC class II molecules which activate the TCR of CD4+ helper T-cells. Depending on the cytokines produced, the adaptive immune response may be geared towards a Th1 response where CTL develop, or towards a Th2 response with a bias towards the induction of antibodies. It is generally thought that a Th1 response is required for effective rejection of cancer cells, although a role for antibodies is not excluded.

In considering MUC1 as an antigen, it is clear that where the response is to the whole molecule, the glycoform is of paramount importance. This will apply to the antibody response, and to lectin mediated interactions with the effector cells of the immune system. MUC1 is a highly repetitive molecule, carrying multiple O-glycans which are different in the cancer mucin. It is therefore not unlikely that this particular glycoform may be capable of acting as a PAMP as well as enhancing cell–cell interactions. Whether the presentation of peptides by MHC molecules is occurring and whether this is affected by glycosylation remains to be clarified. Theoretically, class II presentation could be affected since exogenous antigen is taken up and degraded. However, since class I molecules are loaded in the ER before O-glycosylation is initiated, it would seem unlikely that peptides carrying O-glycans can be presented. The exception may be presentation by dendritic cells

(DCs) which can direct antigen taken up exogenously to the cytoplasm.

As indicated above, the MUC1 mucin was first recognised as a tumour-associated antigen by virtue of its dominance in inducing antibodies in mice. The first indication that the molecule may be immunogenic in humans came from the observations of Finn and colleagues who isolated cytotoxic T-cells (CTL) from breast and ovarian cancer patients which killed MUC1 expressing cells in a non-HLA restricted fashion [55]. These studies indicated that only the carcinoma associated glycoform was recognised by the CTL [56] and it was suggested that the intact molecule made multiple interactions with the T-cell receptors (TCR), the important epitope being found within the tandem repeat [57]. Only later was it reported that antibodies recognising sequences in the tandem repeat are also found in cancer patients [58,59]. These original observations have been followed by studies from several groups working with mouse models and human PBL (peripheral blood leucocytes) attempting to define the cellular responses to MUC1. Classical MHC restricted responses to epitopes within the tandem repeat have been reported for most of the common mouse strains [60]. HLA restricted CTL have also been isolated from HLA-A2 transgenic mice [61] while human CTL able to recognise MUC1 peptide epitopes presented by HLA-A11 have also been recognised [62]. Proliferative responses to the MUC1 tandem repeat peptide possibly representing a helper T-cell response have been reported [63].

In contrast to the studies directed towards understanding and enhancing the immune response to MUC1, inhibitory effects of the mucin on T-cell responses have been reported [64,65]. Clearly, the possibility of the dual function of repelling cells by the highly charged extended mucin or enhancing adhesion via lectin interactions bring a complexity to the function of the molecule in the context of cell interactions which is not easily resolved.

4. MUC1 in immunotherapy of breast cancer patients

4.1. Antibody therapy

The monoclonal antibodies which have been raised to MUC1 have been available since the early 1980s and have been used in the clinic mainly as carriers of radioactive elements. The HMFG1 antibody (originally called 1.10.F3) was developed in 1981 and has been used mainly in ovarian cancer patients to deliver a high dose of yttrium to the peritoneum [66]. In patients with minimal residual disease after chemotherapy, a significant survival benefit was seen as compared to historical controls. The results are of sufficient interest to have prompted a phase III multicentre trial in these patients which is now ongoing. Recently, a small trial has been initiated with unlabelled humanised HMFG1 antibody for adjuvant therapy of breast cancer patients (D. Miles, personal communication) since studies in other cancers have shown that antibody alone can inhibit metastatic growth [67]. Another monoclonal antibody, BrE-3 labelled with ^{111}In has been used to target breast carcinomas [68], and more recently ^{111}In and ^{90}Y-labelled BrE-3 has also been used in the treatment of breast cancer [69]. In this study of six patients, three showed transient clinical response, thus warranting further studies. Imaging studies with radiolabelled antibodies, particularly ovarian cancer patients with the SM3 antibody have shown that malignant tumours can be distinguished from benign lesions. This form of screening could be implemented in high-risk patients, for example, those with a strong family background of disease. Some success has also been observed in detecting breast cancer cells in involved lymph nodes in breast cancer patients [70]. Finally, antibodies to MUC1 are also used in breast cancer management to detect MUC1 in serum in an attempt to detect relapse before clinical symptoms appear.

Antibodies to MUC1 initiated interest in the MUC1 mucin and also led to the cloning of the gene coding for the core protein. Now that the mucin is well characterised, interest in using MUC1 based formulations for active specific immunotherapy of carcinomas expressing the molecule has developed. There are several reasons why the mucin is being seriously considered as a target antigen, not least of which is the fact that both cellular and humoral responses have been observed in cancer patients. Changes in intracellular distribution of MUC1 which is apical in normal glandular epithelia, but distributed all over the surface of the cancer cell, would

allow selectivity in any killing dependent on reaction with the whole molecule. As indicated above, the glycoform of the molecule has to be considered.

4.2. Active specific immunotherapy based on MUC1: animal models

Before clinical studies can be initiated, some form of preclinical testing in animal models is necessary. To this end, syngeneic and transgeneic mouse models have been developed [71–73]. Syngeneic models have been used to investigate the efficacy of immunogens based on MUC1, including naked DNA [71], viral vectors [74], peptides [75] and liposome encapsulation of peptides [76]. Using MUC1 transgenic mice it has been shown that tolerance to the mucin can be overcome without inducing autoimmunity [77,78]. However, even when using MUC1 transgenic mice the immune response observed is representative of the murine repertoire. Studies using HLA-A2 transgenic mice have shown that there are functional CTL-A2 epitopes within MUC1 [61] and probably the next generation of preclinical testing should involve the use of MUC1, HLA double transgenic mice. However, even in this model other compartments of the immune response, eg receptors on APCs are still murine and so no animal model can truly predict the response that will be observed in humans.

4.3. Active specific immunotherapy based on MUC1: clinical studies

Some of the humoral and cytotoxic immune responses to MUC1 in cancer patients are known to be directed to specific sequences in the tandem repeat. As a result, and because there are many pragmatic advantages in using relative short defined amino acid sequences, the clinical studies involving the use of peptides as the immunogen have concentrated on the tandem repeat of MUC1. Phase I studies have reported the use of tandem repeat peptides coupled to diphtheria toxoid [79], BCG [80] or KLH [81]. In the Biomira trial [80], class I restricted CTL were demonstrated after immunisation with 16 amino acids from the tandem repeat coupled to KLH, and increase in CTL precursor frequency has been reported when patients have been

immunised with five tandem repeats mixed with BCG [80].

One formulation that looked very promising in the animal model was the use of a MUC1 tandem repeat fusion protein coupled to oxidised mannan, which should target mannose receptors on APCs. However, although a predominantly CTL response was demonstrated in mice [82], early clinical trials conducted in patients with advanced breast and colorectal cancer show that in the clinical setting the predominant response was humoral, with few CTL being observed [83]. This apparent discrepancy highlights the limitations of translating studies in animal models into the clinical setting. The different response in humans is attributed to the presence of cross reactive antibodies present in humans (but not in mice) which form an immune complex with the antigen, which in turn induces a Th2 type response [84].

The use of tandem repeat peptides as immunogens has the disadvantage that the response is being restricted to one domain of the mucin. One way to overcome this is the use of viral vectors carrying DNA encoding MUC1 which also have the advantage of infecting the cells and so targetting MUC1 into the class I pathway. A recombinant vaccinia virus carrying cDNA for MUC1 and for IL-2 (VV-MUC1/IL2) has been constructed [85] and shown to induce CTL in mouse models. This construct has been used in a phase I study of patients with advanced breast cancer. Immunisation was not associated with significant toxicity and immune responses were detected in some patients [86]. A phase II multicentre trial using the VV-MUC1/IL2 construct in patients with metastatic breast cancer is now in progress.

A direct way to exploit the aberrant glycosylation of MUC1 for immunotherapy is the use of tumour-associated carbohydrate antigens which are found on MUC1, but may also be carried on other glycoproteins. A prospective, randomised clinical trial using STn in patients with breast cancer has recently been reported [87]. All patients were immunised subcutaneously with STn conjugated to KLH, with DETOX adjuvant. Patients were also randomised to receive before the first immunisation either low dose intravenous cyclophosphamide or no or oral cyclophosphamide [88]. This was because it has been suggested that soluble antigen in the circulation can be immu-

nosuppressive and that at least in mice, this can be overcome by pretreatment with low dose cyclophosphamide [89,90]. The treatment had minimal toxicity and the highest antibody titres were in patients pretreated with intravenous cyclophosphamide. Furthermore, the median survival for the group pretreated with i.v. cyclophosphamide was significantly longer than that for the other groups. As there were no differences between the groups in terms of the natural history of their disease or the number and type of previous treatments, the results suggest a therapeutic effect for pretreatment with i.v. cyclophosphamide followed by immunisation with STn-KLH. A large multicentre trial comparing i.v. cyclophosphamide and STn-KLH/DETOX-B with i.v. cyclophosphamide and KLH/DETOX-B in the treatment of patients with breast cancer is about to commence.

5. Summary

In summary, MUC1 is upregulated and aberrantly glycosylated in many carcinomas. It is useful as a model glycoprotein in the study of glycosylation changes associated with malignancy, as changes in activities of glycosyltransferases have been directly correlated with changes in the structure of glycans attached to MUC1. Furthermore, it has many characteristics that make it an excellent candidate molecule for active specific immunotherapy, and this is reflected by the number and scope of early clinical studies that have been initiated.

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